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Benzothiadiazole affects the leaf proteome in arctic bramble (*Rubus arcticus*)

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SUMMARY

Benzothiadiazole (BTH) induces resistance to the downy mildew pathogen, *Peronospora sparsa*, in arctic bramble, but the basis for the BTH-induced resistance is unknown. Arctic bramble cv. Mespi was treated with BTH to study the changes in leaf proteome and to identify proteins with a putative role in disease resistance. First, BTH induced strong expression of one PR-1 protein isoform, which was also induced by salicylic acid (SA). The PR-1 was responsive to BTH and exogenous SA despite a high endogenous SA content (20–25 μg/g fresh weight), which increased to an even higher level after treatment with BTH. Secondly, a total of 792 protein spots were detected in two-dimensional gel electrophoresis, eight proteins being detected solely in the BTH-treated plants. BTH caused up- or down-regulation of 72 and 31 proteins, respectively, of which 18 were tentatively identified by mass spectrometry. The up-regulation of flavanone-3-hydroxylase, alanine aminotransferase, 1-aminocyclopropane-1-carboxylate oxidase, PR-1 and PR-10 proteins may partly explain the BTH-induced resistance against *P. sparsa*. Other proteins with changes in intensity appear to be involved in, for example, energy metabolism and protein processing. The decline in ATP synthase, triosephosphate isomerase, fructose bisphosphate aldolase and glutamine synthetase suggests that BTH causes significant changes in primary metabolism, which provides one possible explanation for the decreased vegetative growth of foliage and rhizome observed in BTH-treated plants.

INTRODUCTION

Arctic bramble (*Rubus arcticus* subsp. *arcticus* L.) is a northern *Rubus* species grown for its high-value, aromatic fruits in Finland. Markets for the fruits are stable or increasing, but the cultivation areas have remained small due to widely occurring downy mildew disease caused by *Peronospora sparsa* Berk. The disease initially appears as reddish, angular, sporulating lesions on the leaves of *Rubus* plants, but the most severe consequence is the drying of immature fruits, which causes high annual fluctuation in yields (Lindqvist *et al.*, 1998; McKeown, 1988). None of the present arctic bramble cultivars is completely resistant to the disease, although differences between cultivars exist (Hukkanen *et al.*, 2008). Two of the oldest cultivars, Pima and Mespi (Ryynänen and Dalman, 1983), are both considered to be susceptible in the field, but recent observations from a greenhouse experiment indicate that cv. Mespi is more resistant to *P. sparsa* than cv. Pima (Hukkanen *et al.*, 2008). A wider selection of cultivars showing resistance will be needed for cross-pollination and to retard disease outbreaks, which may readily occur in cultivations based on only two related cultivars, such as cvs Mespi and Pima. No information is available regarding the molecular basis for downy mildew resistance in arctic bramble that could facilitate breeding.

Benzothiadiazole (BTH) is a plant activator, which is known to induce resistance against a wide spectrum of pathogens by acting as a functional analogue of salicylic acid (SA) (reviewed by Oostendorp *et al.*, 2001). Although the efficacy of BTH has been unquestionably proved in several plants, no clear concept has been established about how the resistance is developed. Most reports describe a connection between pathogen resistance and the accumulation of pathogenesis-related (PR) proteins, mainly PR-1, PR-2, PR-3 and PR-5, or certain defence-related enzymes involved in the removal of oxygen radicals and synthesis of phenylpropanoids (Brisset *et al.*, 2000; Friedrich *et al.*, 1996; Lawton *et al.*, 1996; Ziadi *et al.*, 2001). However, recent findings suggest that induced resistance by pathogens or externally applied chemicals affect plant metabolism broadly, pertaining to primary as well as to secondary metabolism. The analysis of differentially expressed expressed sequence tags from cocoa during early response to BTH revealed up-regulation of 68 different genes, including those participating in defence, photosynthesis,

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protein processing and transport, transcription, and cellular communication and organization (Verica *et al.*, 2004). Comparable analysis on coffee plants showed up-regulation of 55, mainly defence-related, genes and down-regulation of 16 genes in the leaves (De Nardi *et al.*, 2006). A larger fraction of genes was down-regulated in the roots, most of them related to primary metabolism. The third study describing BTH-induced changes at transcript level showed up-regulation of 9–24 genes in wheat, the number of affected genes depending on the sampling time (Pasquer *et al.*, 2005). Most of those genes were related to defence and were, for example, PR proteins or enzymes from the phenylpropanoid pathway. Interestingly, chitinase was the only gene found to be up-regulated by BTH in all of these three studies. The lack of overlap between the studies may be due to varying responses between plant species and different sampling times as well as the limitations of the screening methods available to detect all changes caused by BTH.

Proteomic profiling has become a widely used option for the analysis of metabolic changes related to, for example, plant–microbe interactions and plant defence (Curto *et al.*, 2006; Rajjou *et al.*, 2006; Tsunezuka *et al.*, 2005). However, a wide-scale proteomic approach has not been applied to study BTH-induced defence responses. The benefit of protein-level studies is that they can reveal post-transcriptional regulation or modifications and give an overview that is more directly linked to gene function than is transcript profiling.

In our previous study, BTH was found to induce high-level resistance to *P. sparsa* in arctic bramble (Hukkanen *et al.*, 2008). BTH also induced the accumulation of phenolic secondary compounds in arctic bramble, but the relatively small change in phenolics, particularly with higher concentrations of BTH, is alone unlikely to be responsible for the observed improvement of disease resistance. In the present study, arctic bramble cv. Mespi was treated with BTH in order to identify BTH-affected proteins with putative roles in the development of resistance to downy mildew disease. Protein expression was analysed 4 days after the BTH treatment using two-dimensional protein electrophoresis (2-DE) followed by identification with mass spectrometry. Expression of PR-1 was analysed in Western blots after water, BTH and SA treatments. Furthermore, endogenous SA levels and vegetative growth were determined after BTH treatment.

RESULTS AND DISCUSSION

Growth reduction by BTH

Activation of plant defence by BTH without pathogen challenge can deplete energy from vegetative growth and lead to reduced plant size or yield, as observed in wheat and cauliflower (Godard *et al.*, 1999; Heil *et al.*, 2000). The weight of foliage and rhizome were determined in outdoor-grown arctic bramble after two seasons of successive BTH applications. Significant reductions of 30 and 40% in the weight of foliage and rhizome, respectively, were found, indicating that BTH negatively affects primary metabolism in arctic bramble (Fig. 1A). The reduction in growth was apparently cumulative, as the growth measurements performed during the early season did not indicate slower growth for BTH-treated plants (data not shown). Preliminary observations indicated that flowering and cropping are not decreased by BTH despite the reduction in plant size. BTH, if acting like SA in plants, may affect the direction of metabolism similarly to SA. SA can induce transitions from vegetative to reproductive growth and its accumulation is related to early flowering (Dietrich *et al.*, 2005; Jin *et al.*, 2008; Martínez *et al.*, 2004).

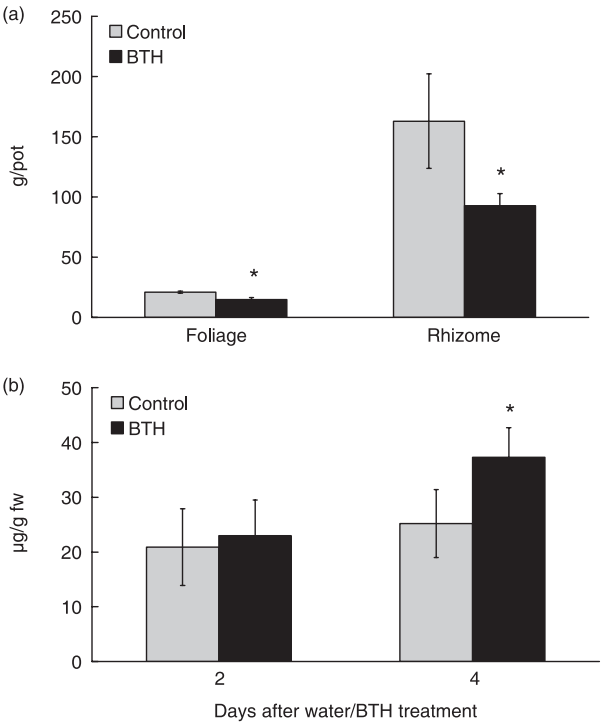

Fig. 1 Influence of BTH treatment on vegetative growth and salicylic acid content in cv. Mespi. (a) Dry weight of foliage and fresh weight of rhizome at the end of season after two successive years of water or BTH treatments. (b) Total SA content measured 2 and 4 days after water or BTH treatment. Statistically significant differences between control and BTH-treated plants are marked with an asterisk ($P < 0.05$; two-tailed Student's *t*-test).

Influence of BTH treatment on endogenous SA levels and PR-1 expression

To explore the basal SA level and its responsiveness to BTH in arctic bramble, free and conjugated SA were measured from the leaves of cv. Mespi 2 and 4 days after BTH treatment. The results are expressed as total SA (Fig. 1B), as the changes caused by BTH

treatment were observed consistently in conjugated SA, which constitutes *c.* 90% of all SA in arctic bramble. The level of total SA increased 1.5-fold in BTH-treated plants between the sampling times, whereas no change was detected in the controls (Fig. 1B). In most other plants, no significant increase in endogenous SA by BTH has been reported and therefore BTH is considered to act as SA or downstream of SA in plants (Friedrich *et al.*, 1996; Lawton *et al.*, 1996). However, the relatively slow accumulation of conjugated SA in arctic bramble may not be connected to BTH-induced signalling. Arctic bramble, similarly to, for example, strawberry, which also showed an increase in conjugated SA after BTH treatment (Hukkanen *et al.*, 2007), is one of the most SA-rich plants studied. These high-SA plants contain much higher levels of endogenous SA than is generally needed for the activation of defence genes in other plants and thus they may also use SA for purposes other than signalling. SA can possibly act as an antioxidant, as observed in rice (Yang *et al.*, 2004), and its function can thus resemble that of other phenolic compounds. This is supported by concomitant accumulation of SA and other phenolic compounds by BTH in arctic bramble and strawberry (Hukkanen *et al.*, 2007, 2008).

The expression of PR-1 protein has been used as a marker for the activation of the SA-mediated defence pathway, although different PR-1 isoforms may have distinct biological functions and patterns of induction (Cameron *et al.*, 1999; Lawton *et al.*, 1996; Malamy *et al.*, 1990; Van Loon *et al.*, 2006). To address the question of whether BTH and SA activate the PR-1 protein (EU528030) in arctic bramble, PR-1 expression was followed by Western blot analysis in several experiments. One PR-1 protein isoform was inducible by BTH, induction being maximal 4 days after the treatment (Fig. 2a). The other two bands detected with the PR-1 antibody were also present without BTH treatment. Figure 2(b) shows the induction of the same PR-1 protein isoform by SA 4 days after the treatment. The result suggests that BTH may activate the SA-mediated defence pathway in arctic bramble. However, we cannot exclude the possibility that other signalling pathways are also involved in the action of BTH, such as the ethylene pathway. As shown below, BTH increased the amount of an ethylene synthesis enzyme, 1-aminocyclopropane-1-carboxylate (ACC) oxidase, previously reported to take place in bean (Iriti and Faoro, 2003). Thus, the action of BTH may also proceed via ethylene at least in some cases, such as in Arabidopsis mutants deficient in the SA signalling pathway, where the action of ethylene was found to be necessary for the induction of PR-1 (Nandi *et al.*, 2003).

The young, not yet fully expanded leaves showed weaker expression of PR-1 by BTH than did the full-size, mature leaves (Fig. 2c). Older leaves also had constitutively higher levels of another isoform of PR-1 than did the young leaves (Fig. 2c, uppermost band). Consistent with our data, mature leaves of rice have been found to express higher PR-1a levels than juvenile

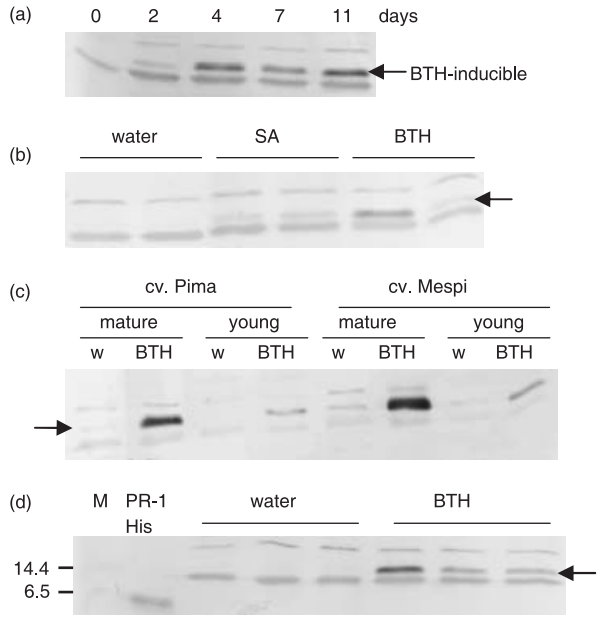

Fig. 2 Western blot analysis of PR-1 protein in arctic bramble leaves treated with water, BTH or SA. (a) PR-1 expression in cv. Mespi 0, 2, 4, 7 and 11 days after BTH treatment. The BTH-inducible band is marked with an arrow. (b) PR-1 expression in two replicate water-, SA- or BTH-treated plants 4 days after the treatments. (c) PR-1 expression in mature and young, not fully expanded leaves of two arctic bramble cultivars 4 days after water (w) or BTH treatment. Older leaves were more responsive to BTH treatment, which can be seen as stronger expression of PR-1 (the band in the middle). The topmost band was also always stronger in mature leaves despite the treatment, indicating age-related expression. (d) PR-1 expression in replicate water- or BTH-treated cv. Mespi plants 4 days after the treatments. The same extracts were used as for 2-DE analysis. PR-1-His peptide (20 ng) was used as a positive control (M_r as kDa).

leaves during pathogen infection, which also correlated with disease resistance (Ponciano *et al.*, 2006). Analysis of three individual plants from both control and BTH treatment showed uniform induction of PR-1 in all BTH-treated plants (Fig. 2d). The same extracts were also used for the analysis of total proteome.

Changes in leaf proteome caused by BTH treatment

To study the effects of BTH on total leaf proteome of arctic bramble cv. Mespi, 2-DE analysis was performed for protein samples collected 4 days after water (control) or BTH treatment (Fig. 3). A total of 792 valid spots were detected at the pH range 4–7. Altogether, 103 spots were up- or down-regulated by BTH treatment, corresponding to 13% of the total number of spots detected. The most evident change was observed in eight proteins, which were detected only in the BTH-treated plants (Fig. 3). Moreover, 27 proteins showed at least a two-fold increase, 37 proteins less than a two-fold increase, seven proteins at least a two-fold decrease and the remaining 24 proteins less than a two-fold decrease by

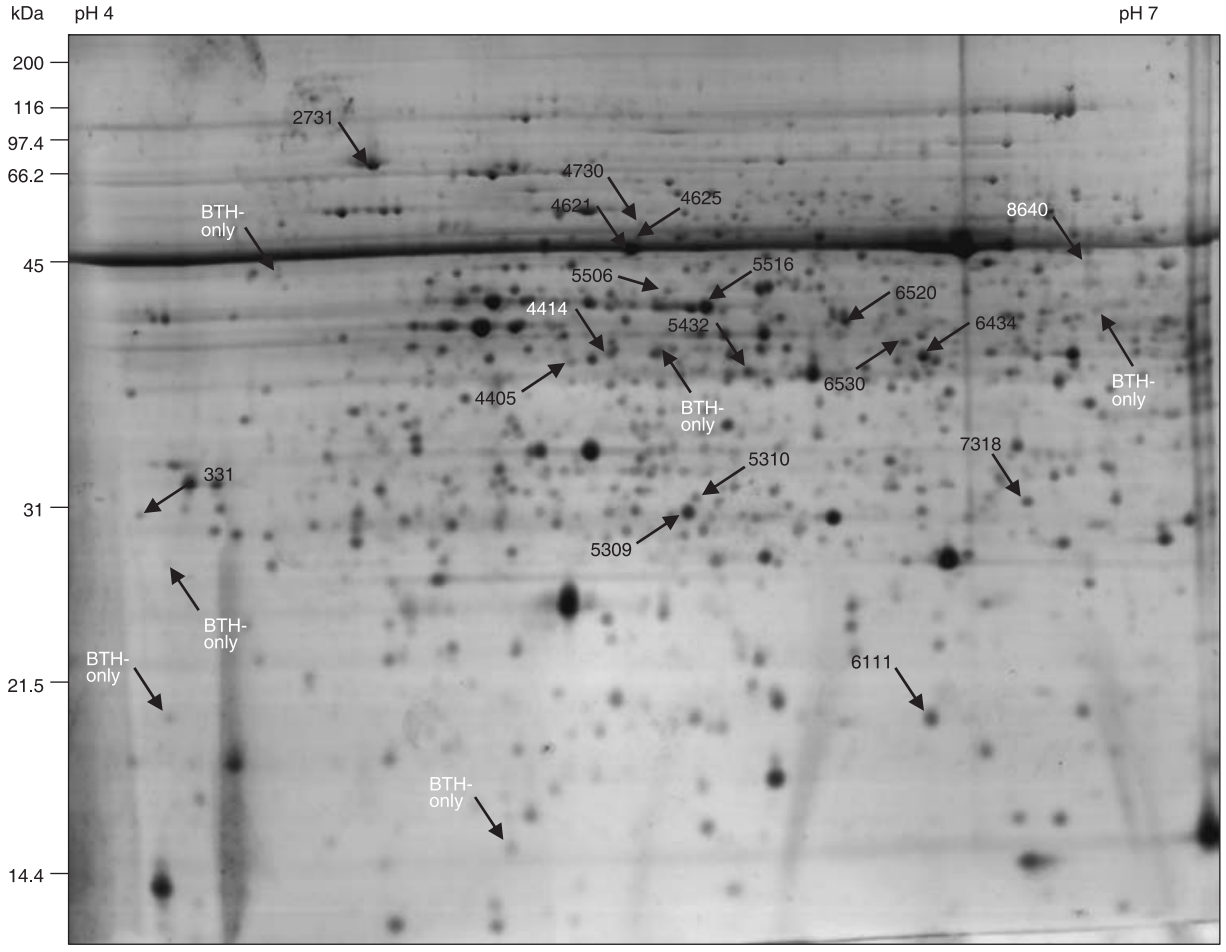

Fig. 3 2-DE separation of SYPRO Ruby-stained leaf proteins from BTH-treated cv. Mespi 4 days after the treatment. Spots with arrows and numbers were identified (see Table 1 for identified proteins). Proteins detected only in the BTH-treated plants are marked in white and with the label 'BTH-only' if not identified.

BTH. Taken together, BTH caused at least a two-fold change in 42 proteins (5.3% of all spots). This result is in agreement with those from transcript profiling studies on cocoa or coffee, where high numbers of genes were affected by BTH treatment (De Nardi *et al.*, 2006; Verica *et al.*, 2004). The spots that showed significant increase or decrease in intensity after BTH treatment were subjected to MS analysis for identification. As practically no information about the protein sequences of arctic bramble exists, the identification was based on peptide matches and homology to proteins characterized in other species. Table 1 shows the 18 proteins that could be tentatively identified. Two proteins were identified based on only one matched peptide (spots 4414 and 5310), but their identification is supported by the previously observed changes at the metabolite level (Hukkanen *et al.*, 2008).

Defence-related proteins up-regulated by BTH

Some of the proteins identified are associated with defence metabolism of plants. Spot 6111, which shows a nine-fold increase by BTH treatment, was identified as a PR-10-like protein, the closest match being a putative allergen Rub i 1 from *Rubus idaeus* (Table 1). The Rub i 1 closely resembles ribonuclease 1 from ginseng and Mal d 1 from apple, both being characterized as PR-10 proteins (Moiseyev *et al.*, 1997; Poupard *et al.*, 2003). The induction of PR-10 protein was also detected in one-dimensional Western blot analysis using an antibody developed against PR-10c of birch by Koistinen *et al.* (2002) (data not shown). The exact function of the PR-10 protein family is obscure and the proteins have shown both developmental and defence-related expression (De Nardi *et al.*, 2006; McGee *et al.*, 2001; Poupard *et al.*, 2003; Swoboda *et al.*, 1995; Ziadi *et al.*, 2001). There is some evidence for their ability to act as ribonucleases, permeabilize membranes and, in particular, bind small hydrophobic ligands such as flavonoids and plant hormones (Fernandes *et al.*, 2008; Koistinen *et al.*, 2005; Mogensen *et al.*, 2007; Park *et al.*, 2004). The suggested role as a carrier protein of flavonoid-type small molecules is supported by the finding made by Hjernø *et al.* (2006), who observed parallel down-regulation of Bet v 1-type

Table 1 Tentatively identified differentially expressed proteins in water- or BTH-treated leaves of arctic bramble.

Spot	Putative ID	Top hit	Peptides	Matched peptides/ coverage (%)	Mass (kDa) Th/Ex*	pI Th/Ex	Normalized intensity (p.p.m. ± SD)		
							Water	BTH	Ratio†
Up-regulated proteins									
331	Nascent polypeptide-associated complex alpha chain	T03926; Q7XXR8	DIELVMTQAGVSR/SPTSDTYVIFGEAK/IEDLSSQLQTQAAEQFK	3/21.5	22.3/30	4.3/4.2	278 ± 102	874 ± 127	3.1
4405	1-Aminocyclopropane-1-carboxylate oxidase	Q4JKT2	SHTDAGGIILLFQDDK/LAEELLDLFCENLGLEK	2/15.7	23.9/38	5.8/5.4	615 ± 285	1367 ± 492	2.2
4414	Flavanone-3-hydroxylase/oxidoreductase	Q9FLV0; NP_197841	IEELISESLGLEK	1/3.8	39.4/39	5.4/5.4	0	1000 ± 189	BTH only
5310	Caffeoyl-CoA-3 *O*-methyltransferase	O23941	ENYELGLPVIEK	1/6.4	21.1/31	5.2/5.7	917 ± 284	1640 ± 339	1.8
6111	Putative allergen Rub i 1 (PR-10-like protein)	ABG54495; P80889; A45786	AFVLDADNLIPK/SVEIIEGDGGVGTVK/ VAPQAXTSVENIYER	2/19.7	14.9/20	8.0/6.3	677 ± 288	6334 ± 4119	9.4
6434	Malate dehydrogenase (cytosolic)	Q946Y0	IVQGLSIDEFSR/LNVQVSDVK/VLVTGAAGQIGYALVR/EFAPSIPEK	4/15.1	35.5/38	6.6/6.3	4021 ± 159	4639 ± 249	1.2
6520	Phosphoglycerate kinase	Q9C7J4; S26623	ADLNVPLDDNQNITDDTR/FSLAPLVPR/ELDYLVGAVSNPK/ GVSLLLPTDVVIADK/GVTTIIGGGDSVAAVEK/LSELLGLQVVK	6/15.1	49.9/41	8.3/6.1	1931 ± 270	2394 ± 92	1.2
6530	Reversibly glycosylated protein	O04300; Q9ZR35	FVDAVLTIPK/VPEGFDYELYNR/DELDIVIPTIR	3/9.1	41.6/40	5.7/6.2	935 ± 71	1631 ± 262	1.7
7318	Proteasome alpha subunit-like protein	Q3HVM0	TTIFSPEGR/DGVVLVGEK/EEAVQLALK	3/10.5	28.1/31	5.4/6.6	2052 ± 252	2641 ± 143	1.3
Down-regulated proteins									
8640	Alanine aminotransferase	Q9S7E9	LLEATGISTVPGSGFGQK/ALDYESLNENVK	2/6.2	53.4/44	6.2/6.7	0	421 ± 34	BTH only
2731	HSP70 (chloroplastic)	Q1SKX2_MEDTR	DIDEVILVGGSTR/GPEGDVIDADFTDSK/AVVTVPAYFNDSQR/ IAGLEVLR	4/7.1	75.8/72	5.2/4.8	3468 ± 347	1764 ± 180	0.5
4621	ATP synthase beta subunit	Q95FL6	ELQDIIAILGLDELSEDDR/GMEVIDTGAPLSVPVGGATLGRI FNVLGEPVDNLGPVDTR/IAQIIGPVLDVAFPPGK/ GIYPAVDPLDSTSTMLQPR/IVGEEHYETAQR/SAPAFIQLDTK/ YVGLAETIR/DVNEQDVLLFIDNIFR/	9/29.8	51.7/47	5.3/5.5	12052 ± 739	8602 ± 1189	0.7
4625	ATP synthase beta subunit	Q95FL6	IGLFGGAGVGK	1/2.3	51.7/48	5.3/5.5	716 ± 173	353 ± 63	0.5
4730	ATP synthase beta subunit	Q95FL6	IFNVLGEPVDNLGPVDTR/ELQDIIAILGLDELSEDDR/ IAQIIGPVLDVAFPPGK/AHGGVSVFGGVGER/ FVQAGSEVSALLGR	5/17.1	51.7/50	5.3/5.5	571 ± 101	306 ± 25	0.5
5309	Triosephosphate isomerase (chloroplast)	Q9M4S8	LVSDLNSAK/LEPDVDVVVAPPFLYLDQVK/GGAFTGEISVEQLK/ RHVIGEDDQFIGK/VASPQQAQEVHVAVR/EEDIDGFLVGGASLK/ GPEFATIVNAVTSK/	7/31.8	33.5/30	7.7/5.7	7297 ± 512	5707 ± 478	0.8
5432	Fructose bisphosphate aldolase (plastidic)	Q9SXX5	LASIGLENTEANR/YAAISQDSGLVPIVEPEILLDGEHGIDR	2/10.3	42.8/38	6.4/5.8	3663 ± 196	3076 ± 72	0.8
5506	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	Q9FXZ7	ETLIAGGPFVLPLAHK/IILTASGGAFR	2/5.7	51.3/43	6.4/5.6	1045 ± 123	806 ± 73	0.8
5516	Glutamine synthetase	Q43127	EEGGFEVIK/GGNNILVICDTWTPAGEPIPTNK	2/7.4	47.4/42	6.4/5.7	9746 ± 949	8218 ± 389	0.8

*Th, theoretical; Ex, experimental value.
†The ratio of normalized quantities, BTH/control. Values below zero indicate down-regulation.

PR-10 protein and the flavonoid pathway in a white-coloured mutant of strawberry. However, the function of PR-10 proteins in defence against pathogens appears to be complicated. The repression of six PR-10-like proteins, which resulted in simultaneous accumulation of a PR-5 protein, improved resistance to the oomycete pathogen *Aphanomyces euteiches* in *Medicago truncatula* although PR-10 proteins normally accumulate during the infection (Colditz *et al.*, 2007).

Consistent with the idea of parallel regulation of the flavonoid pathway and PR-10, two enzymes of the phenylpropanoid pathway were found to be up-regulated by BTH. Flavanone-3-hydroxylase (spot 4414), which catalyses the formation of flavonol precursors, was detectable only in BTH-treated plants. Another enzyme tentatively identified from the phenylpropanoid pathway, caffeoyl-CoA-3 *O*-methyltransferase (spot 5310), was increased 1.8-fold by BTH. The identification and up-regulation of these enzymes in arctic bramble is supported by the previously detected accumulation of phenolic compounds, such as flavonols (Hukkanen *et al.*, 2008). Furthermore, both flavanone-3-hydroxylase and caffeoyl-CoA-3 *O*-methyltransferase transcripts are increased by BTH in wheat (Pasquer *et al.*, 2005). Phenylpropanoids, or phenolics, are associated with both constitutive and induced defence against pathogens in plants (Treutter, 2006) and their accumulation may partly explain the efficacy of BTH against pathogens.

In addition to flavanone-3-hydroxylase, spot 8640 identified as alanine aminotransferase (AlaAT) was detectable only after BTH treatment (Fig. 3; Table 1). In parallel with our observation, Kim *et al.* (2005) reported in hot pepper that AlaAT was inducible by viral and bacterial pathogens, senescence, SA and ethylene and followed a similar induction pattern to PR-1, linking this protein to defence against pathogens. AlaAT may be located in the cytoplasm, mitochondria or, in particular, in peroxisomes, where it catalyses the production of intermediates for photorespiration (Liepman and Olsen, 2003). Interestingly, the activation of photorespiratory enzymes was also found in melon plants over-expressing alanine or serine glyoxylate aminotransferases, which acted as enzymatic *R* proteins against downy mildew pathogen (Taler *et al.*, 2004). In photorespiration hydrogen peroxide is produced which may be used in the activation of downstream defence reactions (Taler *et al.*, 2004). As the cellular location of AlaAT identified in arctic bramble is unknown, its role remains to be elucidated.

ACC oxidase (spot 4405), the last enzyme in the ethylene synthesis pathway, was increased two-fold by BTH treatment. The action of the plant hormone ethylene is considered to be parallel to jasmonic acid rather than to SA, but interfaces for the concerted action of SA or BTH and ethylene have also been detected (Nandi *et al.*, 2003; Rao *et al.*, 2002; Verberne *et al.*, 2003). In Arabidopsis, for example, the accumulation of SA is necessary for the production of ethylene and programmed cell death during ozone stress (Rao *et al.*, 2002). On the other hand, ethylene released during the hypersensitive reaction to tobacco mosaic virus intensifies the expression of SA-inducible PR proteins (Verberne *et al.*, 2003). Ethylene is also needed for systemic acquired resistance and systemic accumulation of SA and PR proteins in tobacco, indicating that ethylene may participate in the generation of a systemic signal in the SA-mediated defence pathway (Verberne *et al.*, 2003). Whether ethylene is involved in the BTH-activated defence is currently unknown. Enzymes of ethylene synthesis have also been found to be induced by BTH and SA treatments, but increase in the end-product, ethylene, has not been confirmed (Iriti and Faoro, 2003; Rao *et al.*, 2002). BTH might prime the production of ethylene by increasing the amount of enzymes, which can be further activated only when necessary for the establishment of other defence reactions. It is possible that in arctic bramble the accumulation of ethylene by BTH causes the slow accumulation of endogenous SA observed in cv. Mespi.

Changes in energy and protein metabolism

Both up- and down-regulation of proteins involved in energy metabolism were found (Table 1). The up-regulated proteins were phosphoglycerate kinase (spot 6520) and malate dehydrogenase (spot 6434), which function in glycolysis and in the citric acid cycle, respectively. The down-regulated proteins were ATP synthase (spots 4621, 4625 and 4730), glutamine synthetase (spot 5516) and the chloroplastic photosynthetic enzymes fructose bisphosphate aldolase (spot 5432) and triosephosphate isomerase (spot 5309). The results parallel those recently reported by Baxter *et al.* (2007). Oxidative stress, which is associated with most defence reactions, was found to enhance catabolic metabolism, glycolysis and the oxidative pentose phosphate pathway, while anabolic processes, such as amino acid metabolism, were reduced. In the present study, the clear decrease particularly in ATP synthase, but also in photosynthetic enzymes and glutamine synthetase, is consistent with the reduction of vegetative growth by BTH (Fig. 1a). Thus far, a biochemical basis for the observed halt in vegetative growth by BTH has not been demonstrated.

Three of the proteins identified are involved in the degradation, folding or transport of proteins. The amount of two proteins was increased by BTH, i.e. nascent polypeptide-associated complex alpha (αNAC; spot 331) and 20S proteasome alpha subunit (spot 7318). αNAC was present at three-fold higher levels in BTH-treated plants compared with controls, whereas the 20S proteasome subunit was only slightly induced. αNAC may function in the targeting and transfer of nascent peptides from ribosomes, in the transport of proteins into mitochondria or in transcriptional regulation (Andersen *et al.*, 2007), whereas the 20S proteasome alpha subunit is part of the large ATP/ubiquitin-dependent 26S proteolytic complex (Ingvardsen and Veierskov, 2001). The activation of protein processing suggests the redirection of protein synthesis and metabolism from primary metabolic routes into defence. In support of our results, 20S proteasome and αNAC have shown

responsiveness to SA and abiotic and biotic stresses in other studies, suggesting a central role for protein processing during defence (Etienne *et al.*, 2000; Ingvardsen and Veierskov, 2001; Rajjou *et al.*, 2006).

Heat shock protein 70 (HSP70; spot 2731) was present in 50% lower amounts in the BTH-treated plants than in the controls (Table 1). The decrease in HSP70 was quite unexpected given that HSPs function in the folding and quality control of proteins together with αNAC and stresses usually increase their quantity (Hartl and Hayer-Hartl, 2002; Kanzaki *et al.*, 2003). The reason for its down-regulation in arctic bramble remains unclear.

Other proteins affected by BTH

One of the proteins up-regulated by BTH was plant-specific reversibly glycosylated protein (RGP; spot 6530). Its function and relation to pathogen defence are unclear, but the protein is presumably capable of self-glycosylation, transporting UDP-sugars, and may be spatially associated with plasmodesmata (Sagi *et al.*, 2005). According to Drakakaki *et al.* (2006), RGPs might function in cell division and in the development of cell walls, for example in pollen. Hence, the increase in RGP may indicate modifications in the cell walls.

1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR; spot 5506), which is a regulatory enzyme in the synthesis of isoprenoid-derived molecules such as chlorophyll, plastoquinones and carotenoids in plastids, was slightly decreased by BTH (Table 1). The isoprenoid pathway has been found to be responsive to abiotic and biotic stresses and to jasmonic acid (Carretero-Paulet *et al.*, 2002; Oudin *et al.*, 2007). The down-regulation of DXR by BTH may reflect the antagonism between SA- and JA-inducible pathways or down-regulation of photosynthesis, but given the high number of different products coming from the pathway, other explanations may also apply.

CONCLUSIONS

Taken together, 103 of 792 proteins were up- or down-regulated by BTH in the leaves of arctic bramble. Although the changes in the quantities of down-regulated proteins were relatively small, a large number of proteins accumulated to more than two-fold higher levels after treatment with BTH. Induction of the defence-associated proteins, namely PR-1, PR-10, ACC oxidase, AlaAT, flavanone-3-hydroxylase and caffeoyl-CoA-3 *O*-methyltransferase, may partly explain the BTH-induced resistance against downy mildew in arctic bramble. The changes in other proteins may reflect the redirection of metabolism to defence, for example in the decline in the enzymes involved in energy metabolism. The reduced vegetative growth of both foliage and rhizome, resulting from repeated application of BTH, might be explained by some of the present findings. Arctic bramble was responsive to BTH and exogenous SA, as shown by the induction of a specific PR-1 isoform, despite very high levels of endogenous SA present in the leaves. The results thus give new information regarding proteome-level changes during induced resistance and may guide future work toward a better understanding of the molecular mechanisms by which BTH protects plants against pathogens.

EXPERIMENTAL PROCEDURES

Plant material and treatments

Arctic bramble cv. Mespi was propagated vegetatively and kept dormant below 4 °C in the greenhouse of the University of Kuopio until the start of the experiment. The plants were grown in peat–sand–vermiculite (14:3:3) under the following conditions: 18 h daylight, 18–22 °C (night–day) and 70% relative humidity. At the beginning of flowering, the plants were sprayed with either water (control treatment) or 0.3 g/L (as active ingredient) BTH (Bion® 50WG, Syngenta) until runoff. After 4 days, three plants per treatment were sampled separately for protein analyses by collecting three full-sized leaves per plant. The leaves were stored at −70 °C. The experiment was repeated for immunoblot analyses under similar conditions using cvs Mespi and Pima. In the repeated experiment, the plants were sampled 2, 4, 7 and 11 days after the treatments. Young and mature leaves were collected separately at 4 days after BTH/water treatment. A set of samples was also collected for the analysis of SA similarly to that described above for proteomics samples. In a separate experiment, arctic bramble cv. Mespi plants were sprayed with water, 2 mM SA (AnalaR, BDH Chemicals Ltd) or 0.3 g/L (as active ingredient) BTH. The plants were sampled as in the previous experiments.

Another experiment with cvs Mespi and Pima was run outdoors for two successive years. The plants were grown in 20-L plastic boxes, each box containing six plants. Four of these boxes were used for both control (water) and BTH treatments. BTH and water were applied to the plants three times per season at approximately 10-day intervals, starting at the beginning of flowering. At the end of the second season, the aerial biomass was collected from each box separately and dry weight was determined. The rhizome from each box was also washed and the fresh weight determined.

Total protein extraction

Frozen samples, each consisting of three leaves from one plant, were homogenized in liquid nitrogen and total soluble proteins were extracted for 2-DE and immunoblot analyses using two-phase TCA/ammonium acetate precipitation as described by Koistinen *et al.* (2002). Protein pellets were dissolved in 2-DE sample buffer containing 10 M urea, 2% CHAPS, 1% DTT and 0.8% Bio-Lyte

3/10 ampholyte (Bio-Rad, Hercules, CA). Protein concentration was measured using Bio-Rad Protein Assay Dye reagent.

2-DE, staining and image analysis

Protein (150 μg) was loaded on three gels for both water and BTH treatments, each gel representing one biological replicate. 2-DE using 24-cm pH 4–7 strips (Amersham Biosciences), staining of gels with SYPRO Ruby fluorescent dye and image acquirement were performed as described by Lehesranta *et al.* (2006). Image analysis was performed with PDQuest 7.1 software (Bio-Rad). Automatic spot detection and matching were used, after which the detected spots were confirmed manually. Before quantification, spot quantities were normalized to the total quantity in valid spots. Normalized intensities of spots were analysed statistically (SPSS software) using a two-tailed Student's *t*-test to identify proteins expressed differentially between the control and BTH-treated plants. Spots having a statistically significant difference ($P < 0.05$) in intensity were selected for MS analysis.

In-gel digestion, MS and protein identification

Silver staining of gels was performed as described previously (Lehesranta *et al.*, 2006). In-gel digestion of excised spots with trypsin was carried out according to Koistinen *et al.* (2002), except that the peptide extracts were dried and dissolved in 25 μL of 0.1% TFA in the final step. Tryptic peptides were analysed using HPLC-ESI-MS/MS as described by Lehesranta *et al.* (2007). Typical ion spray voltage was 2.3–2.4 kV. Myoglobin and ACTH clips were used for the optimization of source parameters and spray position.

Analysis of +2 or +3 charged MS/MS peptides and database searches were performed using Analyst QS v1.1 and ProID software (Applied Biosystems) or Mascot search with Analyst QS. Peptides were identified against the ABCC non-redundant protein database (loaded 23 April 2007) with ProID search and the MSDB (Viridiplantae) database with Mascot search. The following search parameters were used: mass tolerance 0.15 Da; MS/MS tolerance 0.8 Da; one missed cleavage allowed; variable modifications carbamidomethyl (C), carboxymethyl (C) and oxidation (M).

Production of antibody for Western blot analysis of PR-1 protein

To obtain antibody against PR-1, a fragment of 226 bp was PCR-amplified with PR-1-specific primers (containing restriction sites) from genomic DNA isolated from arctic bramble leaves. The whole fragment (GenBank accession no. EU528030) was inserted into the expression vector pQE30 (Qiagen, CA) and the PR-1 peptide containing 6× His-tag was produced, purified and confirmed similarly to that described by Koistinen *et al.* (2002). The antibody was produced in rabbits against the HPLC-purified PR-1-His fragment and purified by affinity chromatography using the PR-1-His coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). The antibody bound to the PR-1-His in the column was eluted with 50 mM diethylamine, pH 11.5, in 1-mL fractions into tubes containing 100 μL of 1 M Tris-HCl, pH 7.4, and 80 mg of NaCl. The fractions showing the highest absorbance at 280 nm were combined and the antibody was dialysed into PBS. The specificity of the antibody was confirmed in Western blots.

Western blot analysis

For Western blot analysis of PR-1 protein, 15 μg of total leaf protein samples was separated by SDS-PAGE at 150 V for 1 h. Three or more parallel samples from each treatment were analysed, each sample representing an individual plant. Two similar gels were always prepared, one for blotting and the other for CBB staining to confirm equal loading of the samples. Blotting and detection were performed according to Koistinen *et al.* (2002). The concentration of diluted primary antibody was 0.2 μg/mL. Goat anti-rabbit IgG conjugated to alkaline phosphatase (1:2000 dilution in TBS-milk) from Zymed was used as the secondary antibody.

SA analysis

SA was extracted with methanol/ethanol, SA conjugates hydrolysed with HCl, extracted into organic solvent and determined by HPLC as described previously (Meuwly and Métraux, 1993). Five parallel samples each from a different plant were extracted separately for both water and BTH treatments.

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